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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
(CUMULATIVE)

(•) Trypsin-like and chymotrypsin-like enzymes have been identified and separated from the digestive tracts of three model insects: the rust red flour beetle Tribolium castaneum, the mealworm Tenebrio molitor and the locust Locusta migratoria. These trypsins and chymotrypsins can be fully inhibited by the proteinaceous trypsin-chymotrypsin inhibitors from legume seeds, such as the Bowman-Birk inhibitor (BBI) from soybeans and CI from chickpeas. (•) The purified and partially-characterized insect trypsins and chymotrypsins differ significantly from the respective mammalian enzymes in their low content of sulfur-containing amino acids in general

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and complete lack of disulfide bridges in particular. This suggests a different three-dimensional structure and assembly of the insect digestive proteases. (•) A revised method with high yield for isolation of the labile trypsin-like and chymotrypsin-like enzymes from Tenebrio molitor larval midgut has been worked out. The method is based on affinity chromatography separation on immobilized, synthetic specific inhibitors which have a very low affinity for the respective enzymes in solution. (•) A technique using polyacrylamide gel electrophoresis (PAGE) that includes gelatin as a substrate was successfully employed for the separation and identification of numerous midgut proteases in Tenebrio and Tribolium. The PAGE-gelatin matrix revealed the inhibitory effect of BBI (the proteinaceous trypsin-chymotrypsin inhibitor from soybeans) on several Tribolium proteases - an effect which was not detectable in inhibition assays in solution. (•) The isolation, characterization and kinetic properties of trypsins and a chymotrypsin-like enzymes from the digestive tract of the locust has been carried out. (•) Comparisons between the insect trypsins and chymotrypsins and their inhibitability by protease inhibitors under different environmental conditions have been drawn. (•) A highly sensitive, specific radioimmunoassay has been developed for the detection and quantitation of trypsin-like enzymes in tissues and insect body fluids using locust-trypsin as a model.

FINAL REPORT
(THIRD ANNUAL REPORT)

PROTEASES OF STORED PRODUCT INSECTS AND THEIR INHIBITION BY SPECIFIC PROTEASE INHIBITORS FROM SOYBEANS AND WHEAT GRAIN

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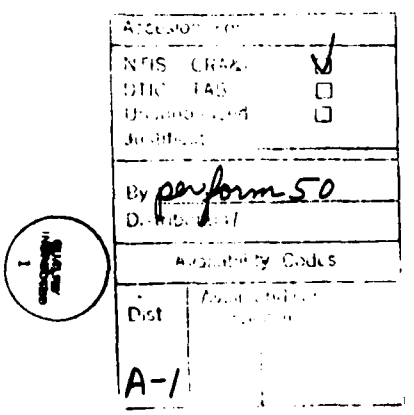

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STATEMENT OF THE PROBLEM STUDIED

A balanced co-evolution is assumed to have developed between herbivorous insects and their hosts, with each type of insect adapting its physiology to best cope with the vagarities of its specific host(s) and the host plant concurrently elaborating methods of defence in order to decrease its suitability for the development of the insect. Stored product insects have evolved a complex relationship with seeds and grains which is conceptually similar to the host-plant relation encountered in herbivorous insects.

Specific protein protease inhibitors, which inhibit digestive protease of insects have evolved in plants. It is often declared that the physiological function of the seed embryo and endosperm protein protease inhibitors is to protect grain against attack by insects and against deterioration by microorganisms.The hypothesis that these protease inhibitors are of potential use in protection of valuable crops from damage as a consequence of attack by insects served as a guideline throughout these studies. The investigation of the digestive proteases of several model insects is a pre-requisite for understanding the complex relationships that the insects have evolved with the plant. The information on insect proteolytic enzymes and more specifically, on insect trypsins and chymotrypsins, in comparison to the corresponding proteases from higher organisms, is essential for studying the selective, species specific, interactions of the naturally-occurring protease inhibitors with the insect proteases.

SUMMARY OF MOST IMPORTANT RESULTS

(•) Trypsin- and chymotrypsin-like enzymes have been isolated from the digestive tracts of three model insects: Tenebrio molitor, Tribolium castaneum and Locusta migratoria and characterized. The inhibitability of these enzymes by proteinaceous trypsin-chymotrypsin inhibitors from soybeans and chick peas suggest that these insects may be affected by the inhibitors in vivo.

(•) The lack of disulfide bridges in the insects' proteases suggest different conformation and assembly from those known for the respective mammalian enzymes. Further investigation of the structure of insect proteases may lead to better understanding of their susceptibility to inhibitors in vivo.

(•) The Bowman-Birk trypsin-chymotrypsin inhibitor from soybeans (BBI) strongly inhibits the trypsin- and chymotrypsin-like enzymes from the digestive tract of the three model insects (Tenebrio, Tribolium and Locusta) when assayed on protein substrates. The affinity of the proteinases for the inhibitors has been increased in immobilized systems, when

compared to their effect in solution. This potentiated effect seems to result from better mutual accessibility of enzyme to inhibitor. It thus may resemble the in vivo interaction between the insect and the raw soybean.

(•) The potential inhibitory effect of soybean protein components on insect proteases has been exemplified by the inhibitability of locust chymotrypsin by the Kunitz soybean trypsin inhibitor (STI) which does not inhibit bovine chymotrypsin.

(•) The Tribolium protease inhibitor from soybeans, which does not inhibit bovine, Tenebrio and locust trypsins, fully inhibits P1, one of the major Tribolium proteases. The proteolytic activity of P1 can also be inhibited by the specific trypsin inhibitors "Kunitz" (STI) and "Bowman-Birk" (BBI) from soybeans. None of these inhibitors affects the Tribolium thiol proteases which have been separated by gel electrophoresis and by affinity chromatography on thio-propylsepharose.

(•) A revised method with high yield has been developed for the isolation of Tenebrio trypsin and chymotrypsin using affinity chromatography on immobilized, synthetic specific inhibitors of trypsin and chymotrypsin, respectively, with very low affinity for the Tenebrio enzymes when assayed in solution. This emphasizes once again the significance of the environment in which the protease "encounters" its inhibitor.

(•) A highly sensitive, specific radioimmunoassay has been developed for the detection and quantitation of trypsin-like enzymes in tissues and insect body fluids using locust trypsin as a model. The specificity of the antibodies was demonstrated by immunoblot technique and cross-reactivity experiments. Positive blot reaction with the antiserum was observed only with the locust trypsin. Bovine porcine, Tenebrio and Tribolium trypsins and locust chymotrypsin did not inhibit the binding of locust trypsin to the antiserum even at 1000-fold concentration.

PUBLICATIONS

Sakal, E., Applebaum, S.W. and Birk, Y. (1988). Purification and characterization of Locusta migratoria chymotrypsin. Int. J. Peptide Protein Res. 29: 590-598.

Sakal, E., Applebaum, S.W. and Birk, Y. (1989). Purification and characterization of Locusta migratoria trypsin. Int. J. Peptide Protein Res. (in press).

Sakal E., Applebaum, S.W. and Birk, Y. (1989). Detection and determination of Locusta migratoria trypsin by radioimmunoassay. (submitted for publication).

PARTICIPATING SCIENTIFIC PERSONNEL

Dr. P. Smirnoff
Isolation and modifiction of protein proteinase inhibitors

R. Golan*
M.Sc. Thesis:

Isolation, characterization and comparative study of proteolytic enzymes from the midguts of Tenebrio molitor adults and larvae as a basis for possible biological pest-control with naturally occurring protease inhibitors from plants sources. (in Hebrew).

E. Sakal*
Ph.D. thesis:

Isolation and characterization of trypsin and chymotrypsin from the digestive tract of the locust Locusta migratoria and their interaction with naturally-occurring and synthetic inhibitors. (in Hebrew).

Y. Saar*
M.Sc. Thesis:

Isolation and characterization of trypsin-like and chymotrypsin-like enzymes from the midgut of Tenebrio molitor. (in Hebrew).

N. Yonah*
M.Sc. (Ph.D. student)
Isolation and characterization of Tribolium proteinases

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*No charge to grant. R. Golan (M.Sc.), E. Sakal (Ph.D.) and Y. Saar (M.Sc.) earned their respective degrees while working on the project

EXPERIMENTAL AND RESULTS

Following is a report on the research carried out during the third year of our project:

(1) A revised method of isolation of Tenebrio molitro trypsin and chymotrypsin by affinity chromatography

One of the major problems in the study of the highly-sensitive Tenebrio molitor proteases has been the very low yield (~ 10%) upon isolation and purification by conventional ion exchange chromatography (e.g. on DEAE-cellulose). Attempts to separate and isolate the respective proteases by reverse-phase or ion-exchange HPLC resulted in full inactivation of the enzymes. The presumed differences in conformation between the Tenebrio trypsin and chymotrypsin, as revealed by lack of disulphide bridges, in comparison to the respective mammalian enzymes has also been noted by the lack of inhibition of Tenebrio trypsin and chymotrypsin by the synthetic specific inhibitors p-aminobenzamidine (PABA) and phenylbutylamine (PBA), respectively. In spite of these findings, an attempt has been made to isolate the Tenebrio enzymes by affinity chromatography on immobilized PABA and PBA. Purified, fully active, trypsin and chymotrypsin, at a yield of 50 - 60% have thus been obtained, indicating once again the importance and significance of mutual accessibility of protease to inhibitor for isolation purposes, and its possible relevance in protection of the seed against stored product pests.

(2) Detection and determination of Locusta migratoria trypsin by radioimmunoassay.

2.a. Introduction

Two trypsin-like enzymes (TLE-1 and TLE-2) have been isolated and purified from the midgut cecae of the locust Locusta migratoria by affinity chromatography and HPLC (First Annual Report). The two enzymes differ in molecular weight and amino acid composition. TLE-1 has a molecular weight of 18kd and TLE-2 24kd. In our attempts to examine the origin and the function of these enzymes, polyclonal antibodies were raised against TLE-2 (the major locust trypsin) and used to develop a highly sensitive and specific radioimmunoassay capable of detecting less than 1 ng of locust TLE-2. We have employed this RIA to measure TLE-2 concentrations in the hemolymph and in several tissue extracts of the locust as well as for immunological relations between the locust trypsins and trypsins from other sources.

2.b. Characterization of TLE-2 antiserum.

Iodinated, HPLC purified, locust TLE-2 ([^{125}I]TLE-2) of high specific radioactivity can be stored at -20°C for four weeks. Fig. 1. shows the results obtained from four different dilutions of the antiserum when varying amounts of unlabelled TLE-2 were added. The amount of the

binding fraction varied inversely with the antibody dilutions. At final dilution of 1:10000 addition of 1 ng of unlabelled TLE-2 almost hardly displaced the radioactive tracer. At 1:25000 addition of 1 ng of unlabelled TLE-2 resulted in 20% displacement, and with 1:50000 and 1:100000 dilutions the displacement was 30 amd 35% respectively. A dilution of 1:50000 was found suitable for assaying 0.5-10 ng of TLE-2 per tube. The respective binding in the absence of unlabelled TLE at antibody concentration of 1:10000 was taken as 100%.

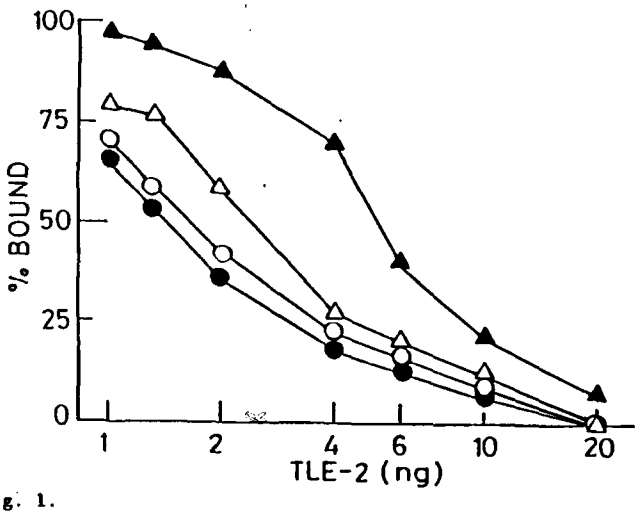

Fig. 1.

Effect of locust TLE-2 antiserum dilutions on [^{125}I]TLE-2 binding. Binding was assayed using four different dilutions of antiserum in the presence of increasing concentrations of unlabelled TLE-2. The final dilutions of the antiserum were 1:10000 (▲) 1:25000 (△) 1:50000 (○) and 1:100000 (●).

2.c. Specificity of locust TLE-2 antiserum.

The specificity of the antiserum was examined by assessing the cross-reactivity of the antiserum with related proteolytic enzymes and by immunoblot. Competition binding assays were performed with trypsins and chymotrypsin from the locust, trypsin and chymotrypsin from bovine and trypsins from hog, Tenebrio molitor and Tribolium castaneum using the standard RIA procedure. Inhibition of TLE-2 binding to the antiserum was observed only with the second trypsin of the locust-TLE-1. None of the trypsins and chymotrypsin from vertebrates displaced the binding of [^{125}I]TLE-2 to the antiserum. Even insects

proteases did not bind to the antiserum in concentration 1000 fold greater than TLE-2. It can be seen from Fig. 2 that TLE-1 inhibits the binding of [^{125}I]TLE-2 to the antiserum 300 fold less effectively than unlabelled TLE-2.

Another determination of the recognition pattern of the antiserum was made by western blot. Fifty µg samples of trypsins from bovine, hog, Tenebrio molitor, Tribolium castaneum, TLE-1, TLE-2 and locust chymotrypsin were run on the blot, only TLE-1 and TLE-2 were recognised by the anti TLE-2 serum.

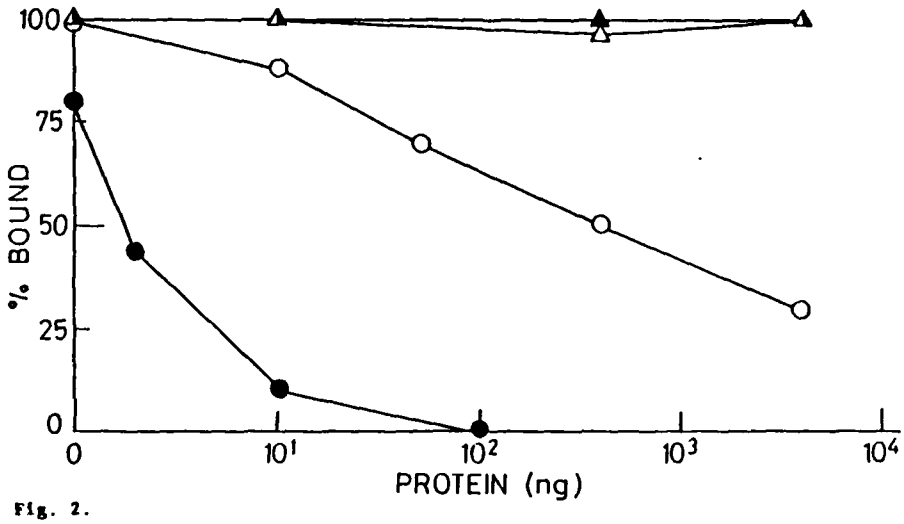

Fig. 2.

Displacement of [^{125}I]TLE-2 by unlabelled TLE-2, TLE-1, trypsin from Tenebrio molitor and trypsin from Tribolium castaneum. Displacement curves were generated by the standard RIA procedure. TLE-2 (●) TLE-1 (○). Tenebrio molitor trypsin (△) Tribolium castaneum trypsin (▲)

2.d. Determination of tissue TLE-2 by RIA.

In an attempt to identify the origin of TLE-2 production , tissue extracts were examined for their immunoreactive trypsin content. The concentrations of TLE-2 in cecal fluid, cecal wall, fat-body and hemolymph, measured by the standard RIA procedure are given in Table 1. The highest immuno activity showed up in the digestive system, where it is in good correlation with the esterolytic activity detected by hydrolysis of tosyl-L-arginine-methyl-ester (TAME). Activity could be detected both in cecal fluid and in cecal wall extracted with 0.1% Triton X-100. The amounts of immunoreactive trypsin found in hemolymph (6.4 ng/mg tissue) and fat-body (0.7 ng/mg tissue) could not be detected in any other way except RIA.

Table 1. Levels of TLE-2 (ng per mg tissue) of cecal fluied, cecal wall, hemolymph and fat-body of Locusta migratoria
Results are means ± SEM based on determination performed in three different preparations obtaind from 20 locusts each.

source	TLE-2 ng/mg tissue	
	detected by RIA	detected by esterolytic activity
cecal fluid	2790 ± 145	1290 ± 280
cecal wall	60.1 ± 5,6	92 ± 12
hemolymph	6.4 ± 0.9	ND
fat-body	0.7 ± 0,08	ND

2.e. Conclusions

The RIA methodology developed for pancreatic endopeptidases is a powerful tool for detection and quantitation of minute amounts of immunoreactive trypsin in insects. The results shown in Fig.1 indicate that [^{125}I]TLE-2 can be used as the radioligand in a RIA procedure which is highly specific and sensitive for quantitative determination of less than 1 ng of TLE-2. The 0.3% cross-reactivity found between TLE-2 and TLE-1 (Fig. 2) is sufficient to detect TLE-1 on immunoblot with TLE-2 antiserum (Fig. 3). The fact that cross-reactivity was found between the two locust trypsins suggests a common ancestry. Although many insects' proteases share common characteristies such as lack of S-S bonds, no cross reactivity was observed between the locust trypsin and trypsins from Tenebrio molitor and Tribolium castaneum, or between TLE-2 and the locust chymotrypsin.

In an attempt to detect the source of synthesis of TLE-2, tissue extracts were examined for their immunoreactive trypsin content. The possibility that midgut enzymes of the insects are produced by the midgut epithelium and are secreted into the gut lumen was examined by extracting the midgut cecal walls with 0.1% Triton. The data in Table 1. indicate a content of 60.1 ng TLE-2 per mg cecal wall. Esterolytic activity was also detected in appriciable amounts in the cecal wall.

The use of an immunological method allows the detection of enzymatically inactive forms of enzymes. The RIA for TLE-2 enables also the detection of the level of immunoreactive trypsin in hemolymph (6.4 ng/mg tissue) and fat-body extracts (0.7 ng/mg tissue) that could not be detected in other ways.

The fact that locust cecal wall extracts contain 100 fold TLE-2 than the fat-body points toward the possibility that trypsin of the digestive system of the insects is produced in the midgut epithelial cells, but does not conclusively prove that this is so. Incorporaion experiments with radiolabelled amino acid precursor are necessary for the definitive conclusions.